PATENTS

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Applicant(s):	Moore et al.	
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RULE 37 C.F.R. § 1.132 DECLARATION
of Barry D. Moore

I, Barry Moore, do hereby declare and say as follows:

1. I am giving this declaration under 37 CFR Section 1.132 in support of the patentability of the invention recited in United States Patent Application Serial No. 10/007,257, filed November 13, 2001. Documents are attached to this declaration as Exhibit A in support of the statements made in this declaration. The content of Exhibit A is incorporated in this declaration by reference.

2. I hold the position of Reader in Chemistry at University of Strathclyde where I have been employed for over 14 years.

3. I have been involved in the field of biophysical chemistry for the past 18 years and am familiar with most aspects of protein stability in non-aqueous solvents, including the formulation and coprecipitation of protein molecules.

4. The above-described patent application is directed to the use of coprecipitants to precipitate biological molecules from solution to form particles having a coprecipitant core.

5. I have supervised Michaela Kreiner who has conducted various experiments to replicate the method disclosed in the Randen reference and then performed various tests to characterize the resultant particles obtained using the Randen method. In addition she has prepared and characterized starch particles obtained using the methods disclosed in the patent. The experiments and tests are shown in Exhibit A.

6. The results of the experiments demonstrate that starch-based particles produced by the Randen method and the method of the patent application are approximately the same. Notably, the starch-protein particles are substantially amorphous, which is shown from the SEM images and the Differential Scanning Calorimeter (DSC) studies. The amorphous structure is not consistent with the existence of a coprecipitant core coated with biological molecules. In contrast, particles made with materials such as amino-acids according to the claimed invention provide sharp DSC melting points that demonstrate high crystallinity.

7. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; further, that these statements were made with the knowledge that willful false statements or the like so made are punishable by fine or imprisonment, or both, under 1001 of Title 18 of the United States Code and that such willful false statement may jeopardize the validity of the application or any patent issued thereon.

BDM
Barry D. Moore

20th August 2004
Date

STARCH-PROJECT

In this study PCMC formation was compared with a co-precipitation method published earlier (L. Randen, J. Nilson, P. Edman. J. Pharm. Pharmacol. 1988, 40: 763-766; R.O. Bustos, C.R. Romo. Chem. Tech. Biotechnol. 1996, 65, 193-199) with the aim of establishing differences between the methods proving the novelty of the production of PCMCs.

The material and method section of the papers above is very vague. The properties of the precipitates, however, may depend strongly on the precipitation conditions. I tried to choose the conditions in order to get a qualitative overview on the effect of conditions on the properties of the precipitates and quantitative data on selected conditions only.

Choice of starch

THEY

Bustos at al. (1996): Maltodextrins PSM 10 and PES 100 corresponded to water soluble isopropyl derivatives of hydrolysed corn starch and were obtained from Reppe Glykos AB, Sweden.
(Note: According to Reppe Glykos AB/Carbamyl AB, Reppal PSM 10 and Reppal PES 100 are both made from potato starch and PES 100 is a derivative of hydroxypropyl starch, so the information in the paper is not correct.)

Randen. et al. (1988): Low molecular mass starch (MW = 12700) and water soluble high molecular mass starch (MW = 100 000), both from Reppe Glykos AB, Sweden.

Note:
(1) PSM 10 and PES 100 have been transferred from Reppe Glykos AB to Carbamyl AB.
(2) According to Carbamyl AB, Sweden, PSM10 is supposed to be very similar to the low molecular weight starch used by Randen et al., though probably not completely the same. It may be that the high molecular weight starch used in 1988 was based on "ordinary" potato starch, and was not a hydroxypropyl derivate, as PES 100 is.

WE, reproducing their method

1) PSM 10 (MW ~ 10 000), kindly provided by Carbamyl AB, Sweden (contact: Hans Rydin, hans.rydin@lyckeby-starch.se)
2) PES100 (MW = 100 000), hydroxypropyl starch, kindly provided by Carbamyl AB, Sweden
3) Paselli SA2 (MW range ~ 12700) kindly provided by Avebe, The Netherlands (contact: Leon Marchall, marchall@avebe.com)

Choice of solvent

THEY

Randen. et al. (1988): acetone, ethanol, 2 -PrOH
Bustos at al. (1996): ethanol (95%)

WE, reproducing their method

mainly EtOH

Theoretical Loading

THEY

Randen. et al. (1988): 0.4 – 16.7 %, if % given in paper means % in aqueous, or 75 –85% if % given means starch per (starch + enzyme)

(paper in detail: Protein concentration of aqueous solution (TRIS, pH = 7.4) : 1 - 30 g /ml (3 – 4.5 ml in total). Starch was added to give a final concentration of 15, 20, 25% (w/w).
Bustos at al. (1996): 50 – 90 %
(paper in detail: 7.2 to 4 mg/ ml of enzyme (in 0.1mol/l borate buffer, pH=8.5). Addition of 0.8 to 4 mg / ml (10 - 50 % w/w) of starch)

WE, reproducing their method

In this study usually 10.8% (see table 1). For comparison, this is the typical loading of standard K_2SO_4-PCMCs

Note: Theoretical loading means initial weight of enzyme per weight of enzyme and carrier used for the precipitation

Aqueous/org. solvent ratio

THEY

Randen. et al. (1988): 1:22 to 1: 33 (Note: they made a batch of 30 g of enzyme, this means they had to use 20 l of solvent according to their method described in their paper!!)
Bustos at al. (1996): 1: 1

WE, reproducing their method

Usually 1:33 (1:15 in PCMC standard method)

Mode of precipitation

THEY

Randen. et al. (1988): The aqueous phase (3 – 4.5 ml) was mixed with the organic phase (100 ml) while stirring at room temperature. The precipitation was rapid.
Bustos at al. (1996): Co-precipitation by adding ethanol and leaving for 1 h at 0°C.

WE, reproducing their method

It was agreed that the method according to Randen et al (1988) would be more relevant for comparison with the PCMC method, because it is more similar to the PCMC method than the Bustos et al. method. Therefore samples in this study, prepared according to Randen et al, were made by adding the aqueous solution of starch and enzyme to the organic solvent, either by pouring quickly or in "1-shot" with a Gilson pipettor (5 ml tip), while stirring (B&T (A Searle Company) - Htplate Magnetic Stirrer, speed 5). In comparison PCMCs are made by adding aqueous solution drop-wise with a Gilson pipettor (yellow tips: 0-200 ul volume). Conditions are summarized in Table 1.

Post-precipitation treatment

THEY

Randen. et al. (1988): Co-precipitates were collected on a Munktell filter. Samples were dried in a vacuum for 72h at 35°C, and milled.
Bustos at al. (1996): Samples were filtered through 4 5um nylon filters, and oven-dried at 45°C to constant weight.

WE, reproducing their method

In this study the samples prepared according to Randen et al. were filtered through a Durapore membrane filter (type HVLPO4700) and dried at 37° C for 3 days. For comparison, PCMCs are filtered through a Durapore membrane filter (type HVLPO4700) and air-dried at RT.

RESULTS

SEM

All preparations (starch precipitated on its own, SC/starch precipitated as in Randen paper, SC/starch precipitated according to PCMC method) resulted in the formation of often roundish-shaped particles. These amorphous-looking masses showed sometimes cracks. The interior of the masses showed pores. On a few occasions (sample 3 and 5) string-like particles were found, which looked similar to the co-precipitate of krill protease and high molecular weight starch described by Randen et al. No crystal-like feature was detected in any of the samples.

See Table 1 for sample details, printed images are in the appendix as well as on file (Starch SEM summary.ppt). SEM was done on samples with PSM 10 and PES100, but not on samples with Paselli starch.

Method:
Dried samples were re-suspended in EtOH (about 6 mg/ml, re-suspending was partly difficult) and dropped onto the sample holder, air-dried and gold-coated. A Jeol JEM 1200 EX transmission electron microscope (Jeol, Tokyo, Japan) was used.

DSC

PSM10: "main" melting point at ~ 260 - 265°C
PES100: "main" melting point at ~ 275°C (285°C and 310°C)
Paselli SN2: ~275°C

For comparison, Sigma gives a melting point of 256 –258 C for their soluble starch.

DSC scans are shown in Fig.1A (PES 100), 1B (PSM10), 1C (Paselli), 1D (control: glycine). Thermogramms of some other preparations (as described in Table 1) can be found in the appendix.

PSM10 *as received* shows a quite well-defined melting point, whereas PES100 shows a wide melting area with possibly up to 3 melting points. The melting points of starch without enzyme are more pronounced than those of co-precipitated preparations. Co-precipitations showed similar DSC curves, regardless if precipitated according to the "PCMC-method" or as in the Randen paper. Peak below 100°C, which appears in all graphs, may be due to dehydration. Control samples known for high crystallinity (glycine as received and PCMC: SC/glycine/EtOH) show sharp melting peaks compared to the starch samples.

The decrease in peak intensity of transitions in the presence of protein indicates strongly that there is intense interaction of protein and starch (mixed in the bulk, not just on surface). However, other factors which might lead to similar results can not be completely excluded.

- Are the multiple melting peaks of starch as received real or are they caused by repeated melting recrystalisation processes? Protein on the surface could affect this process in a way that leads to reduced peak areas, as we found for SC/starch samples. To investigate this, different heating rates were employed (5C/min, 10C/min, 20C/min). If repeated melting re-crystalisation processes were the reason for the multiple "melting points", then the peak areas of the peaks at higher temperatures should decrease with increasing heating rate, because there will be less time for recrystallization. On the other hand, the multiple melting peaks could be the result of the melting of different molecular weights (the ratio of the peak areas should remain constant at different heating rates). With PES100 *as received* it was found that the melting peak at higher temperature is increasing with increasing heating rate (with PSM10 it is a bit difficult to say). So we can exclude repeated melting/recrystallisation for PES100.

- It is also possible that some degradation is taking place during the melting. Thermal gravimetrical analysis (TGA) should give an answer to this (see below).

To summarize, starch is a very complex system and per se far less crystalline than other carriers, like glycine and D,L valine. To be able to give an ultimate interpretation of the DSC results, a lot more has to be studied. From the results we got so far, we may however say it is more likely that the protein is in the bulk of the starch and not only as a coating on the surface, as with the PCMCs using other carrier compounds.

Method:
DuPont Instruments, 910 Differential Scanning Calorimeter
Temperature profile: Heat at 10°C/min from 30°C (or 50°C) to 350°C; N_2 flow: 80 ml/min

Enzyme activity in organic solvent

Subtilisin Carlsberg (SC)

SC/PSM10 PCMC show similar activity as SC/K2SO4 PCMCs, whereas SC/PES100 PCMCs have very low activity. (Fig. 2A). The differences in activity may be related to the high water sorption capability of PES 100.

Method: Transesterification of N-Acetyl-tyrosine ethyl ester
Activity was measured by HPLC following the transesterification of *N*-acetyl-L-tyrosine ethyl ester (10 mM) and 1-propanol (1 M) with acetonitrile/1% H_2O as solvent. Reaction was started by addition of 0.67 mg SC.

Agitation: 150 rpm
Temp: 25C
Assay details:
7.5 mg *N*-acetyl-L-tyrosine ethyl ester
224 ul 1-PrOH
ad 3 ml CH_3CN/1.0 % H_2O

Pseudomonas cepacia lipase (PCL)

PCL/PES100 PCMCs show higher activity than standard PCL/K2SO4-PCMCs. Activity assay for PCL/PSM10 needs to be repeated, as parallel samples show contradictory results. See figure 2B. It would be worthwhile to test if co-precipitation of PES100 has a similar positive on effect on other lipases.

Kinetic resolution of 1-phenylethanol
0.3 M vinylacetate and 0.1 M 1-phenylethanol in dry *tert*-butyl methyl ether. Reaction was started by addition of lipase (1.5 mg, The PCMCs were washed with dry *tert*-butyl methyl ether to remove 1-PrOH just before starting the reaction.)

Agitation: 150 rpm
Temp: 30C
Assay details:
30 ul 1-phenylethanol
69 ul vinylacetate
2.5 ml dry *tert*-butyl methyl ether

Dynamic vapour sorption (DVS)

As shown in Figure 3A, PES100 (hydroxypropyl derivative of potato starch) *as received* absorbs a lot of water (70 % change in mass), which is not surprising. Results for SC/PES100 co-precipitates (preparation no.4 as in Tab. 1; 10.8 % loading, made according to Randen et al. paper) are shown in Fig. 3B. The co-precipitate takes up less water (50 % change in mass) than the control.

Light microsopy

Samples with PES 100 (Fig. 4) and PSM 10 (Fig. 5) are shown.

1. Starch (15% in aqueous) into EtOH (as preparations 10 and 11 in Table 1)
2. SC/starch into EtOH, prec in 1 shot (as preparations 4 and 6 in Table 1)
3. PCMC: SC/starch/EtOH (as preparations PCMC3 and PCMC4 in Table 1)
4. SC/starch into EtOH, drop-wise addition (as preparations 8 and 9 in Table 1)

Result: -needle like structures, particularly with PES 100
-PSM10 particles are much smaller than PES100
Note: samples dry very quickly on microsopy slide, -> inclusions

Method:
Suspensions of co-precipitates were mounted on glass slides and protected by cover slips. Images were produced via a Nikon Optiphot-2 microscope, on which was mounted a Sony CCD video camera (Model XC-77CE).

Thermal gravimetrical analysis (TGA)

PES 100 as received (Fig 6A):
minor change: below 100°C
major change: sharp, starts at ~260°C, ends at about 340°C,
residue of this degrades only slowly.

From this we can conclude that the temperature range of degradation coincides with the "melting" temperature. Therefore it is impossible to say if one of the multiple "melting points" in the DSC is due to degradation

SC as received (Fig 6B):
minor change: below 100°C
major change: sharp, starts at ~260°C, not as sharp as for PES100

Degradation of SC happens at the same temperature as degradation of PES 100. The finding that the melting of starch coincides with the degradation of both, the starch and SC, makes interpretation of DSC results tricky!

Method:
Shimadzu TA 54;
Temperature profile: Heat at 10°C/min from 20°C to 600°C. Hold for 20 min at 600°C
Residual weight (%): (weight in mg at time t)/(weight at time = 0) x 100
Weight at time = 0: PES100 as received (7.737 mg), SC as received (7.654 mg)

Miscellaneous

Solubility of starches in H_2O at RT

Type of Starch	*15% solution*	*20% solution*	*25% solution*
PSM 10	yes	yes	no
PES 100	yes	yes	yes
SN2	no	no	no

no means not soluble even after shaking for > 4h at RT

Visual check: "PCMC" of SC/starch into several organic solvents

Method: 2 mg SC dissolved in 50 ul TRIS (10 mM, pH = 7.8), mixed with 150 ul starch solution (15%, 20%, 25 %), added into 3 ml ethanol (acetone or 2 PrOH) while vortexing (15%, 20%, 25% starch solutions result in a theoretical loading of 8.2 %, 6.3%, 5.1%)

With PSM 10 particles are generally smaller than with PES 100
Particles are smaller in EtOH than in acetone and 2-PrOH
maybe: the lower the starch concentration in aqueous, the smaller the particles
with PES 100: needle-like structures, precipitates is partly clumped together.

Visual test: Does speed of addition of aqueous solution to organic solvent affect particle size?

Method: 300 ul starch solution (15%PSM 10 or PES 100) was added to 12.5 ml EtOH, while vortexing, either by fast pouring or drop-wise using a Gilson pipettor (blue tip: 200-1000 ul volume).

The resulting precipitates of each starch do not show any visual difference

General Note:

PSM10 (powder as received) sticks to spatula, and is therefore difficult to transfer. Randen et al. mentioned grinding problems: difficult to mill owing to adhesion to the mill with low molecular weight starch

Conclusion

With the methods we used to characterise protein starch co-precipitates (mainly SEM and DSC), it appears that co-precipitates made by standard PCMC method and a similar method described earlier (Randen, et al. 1988; Bustos, et al. 1996) are similar. However, as starch is considered a semi-crystalline compound and as such per se far less crystalline than standard carriers used for the PCMCs, starch does not fit the principle of PCMC formation in general. In contrast, Bustos et al. state that only polymeric compounds (hydrolysed collagen, casein and maltodextrins PSM 10 and PES 100) were used in their method because of the nature of the co-precipitation. Therefore, apart from experimental differences, a major difference between these methods lies in the type of co-precipitant, being either a polymer or a low molecular weight compound, like salts, amino acids and simple sugars.

SC/PES100/2PrOH
(Sample 1)
according to Randen et al (pouring),
6.25% loading, PES100 in H2O =15%
MKB1x1a

SC/PES100/2PrOH
(Sample 1)
according to Randen et al (pouring),
6.25% loading, PES100 in H2O =15%
MKB1x4a

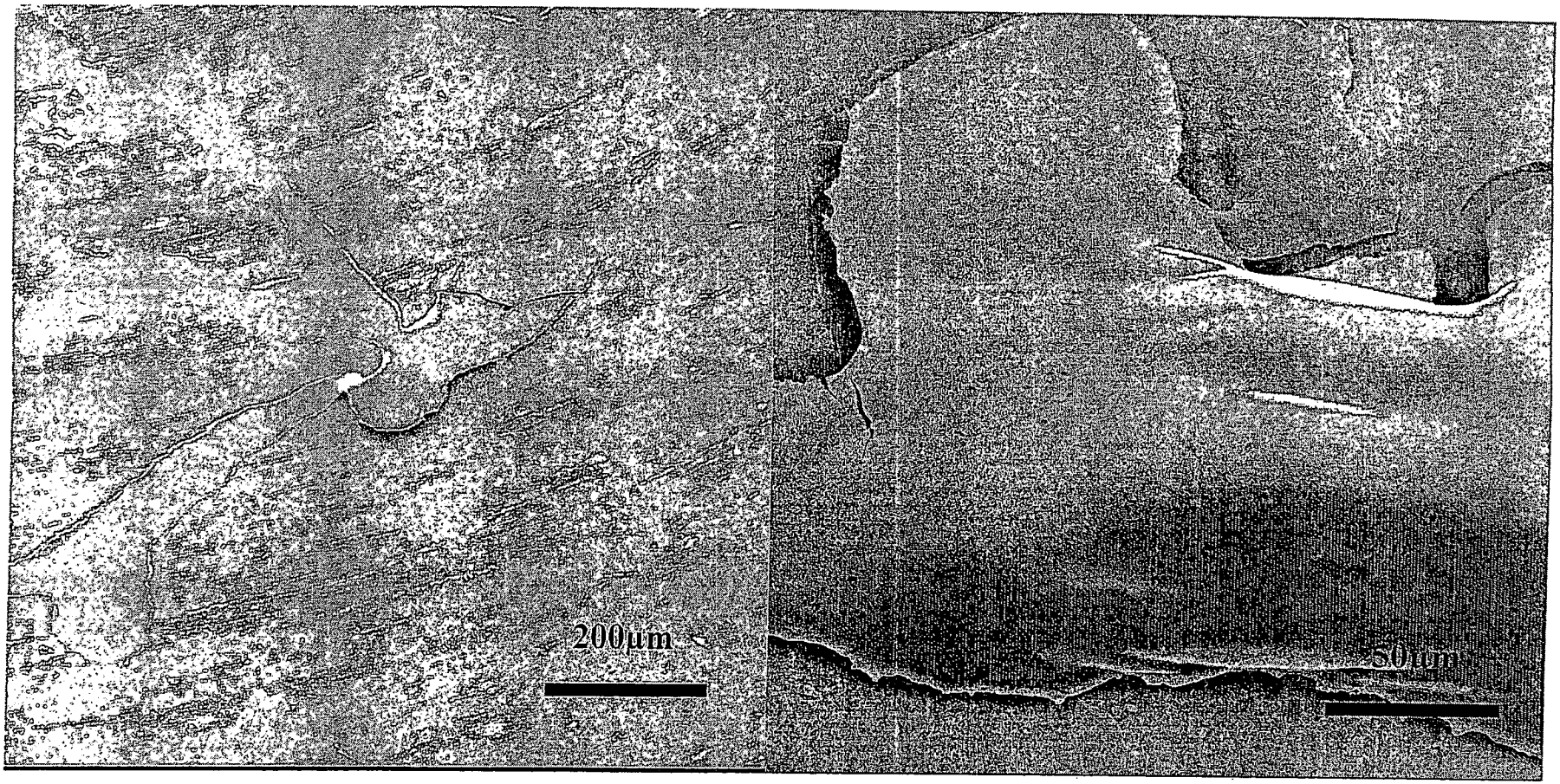


Exhibit A

DSC - results

Fig. 1A PE100 samples

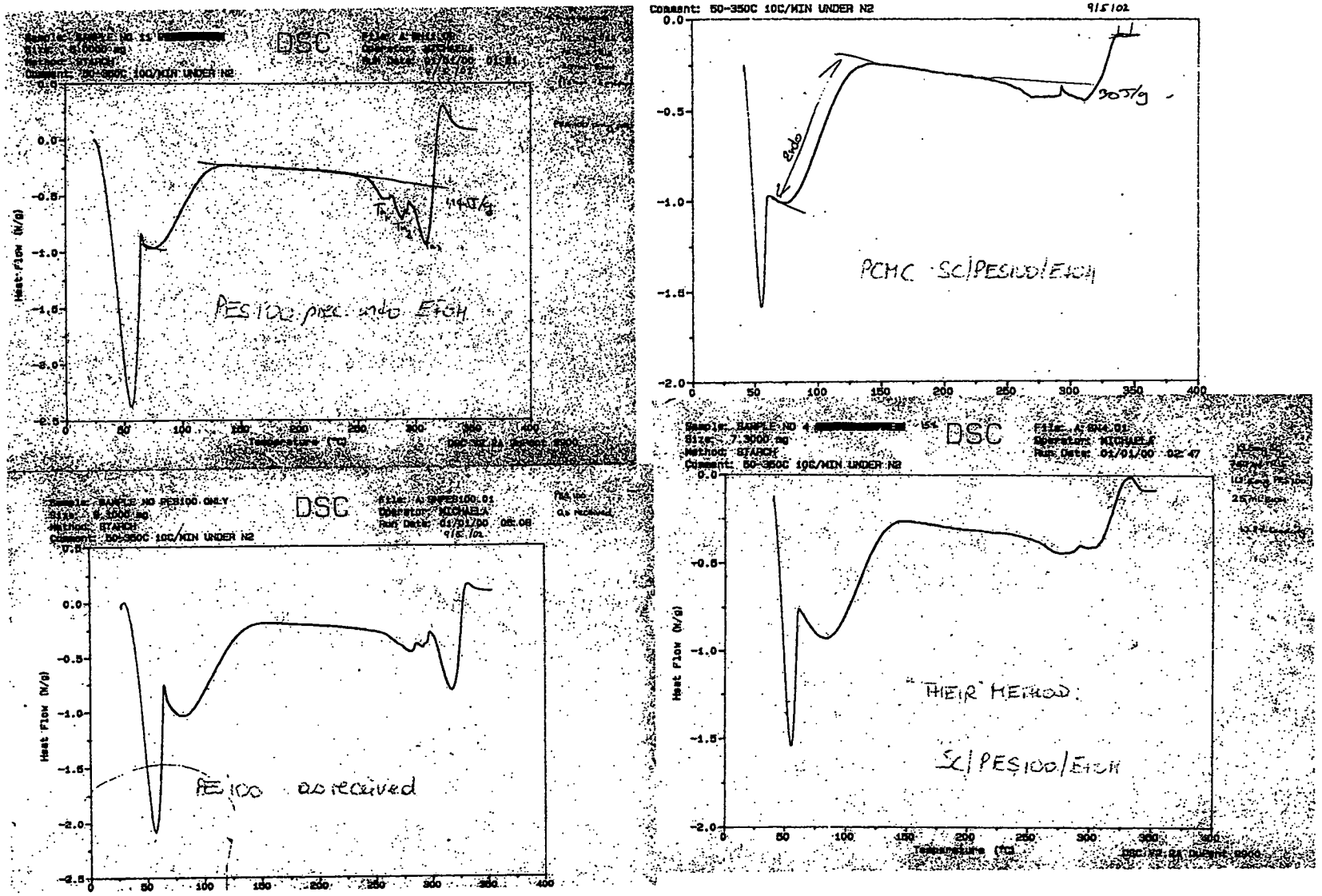
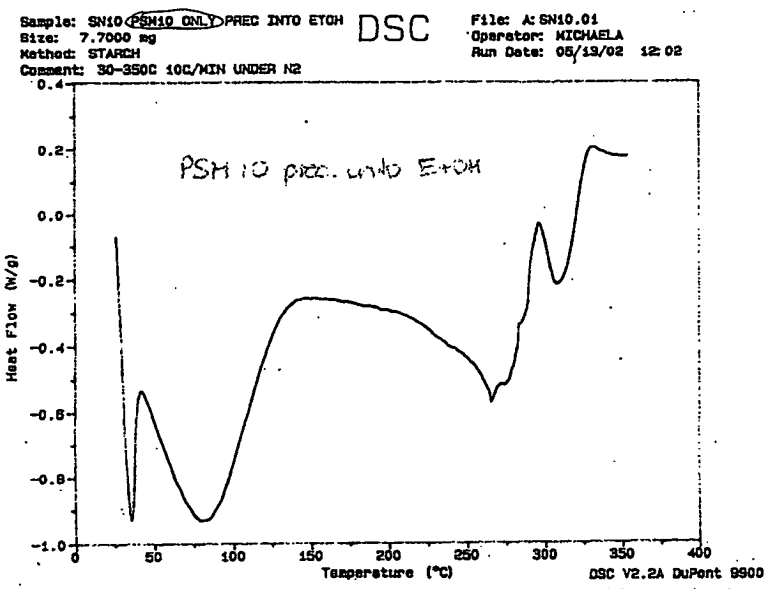


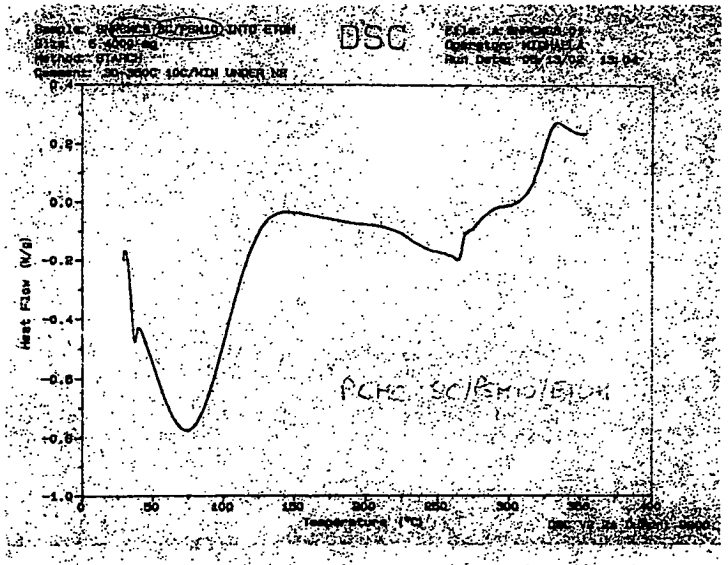
Fig. 1A

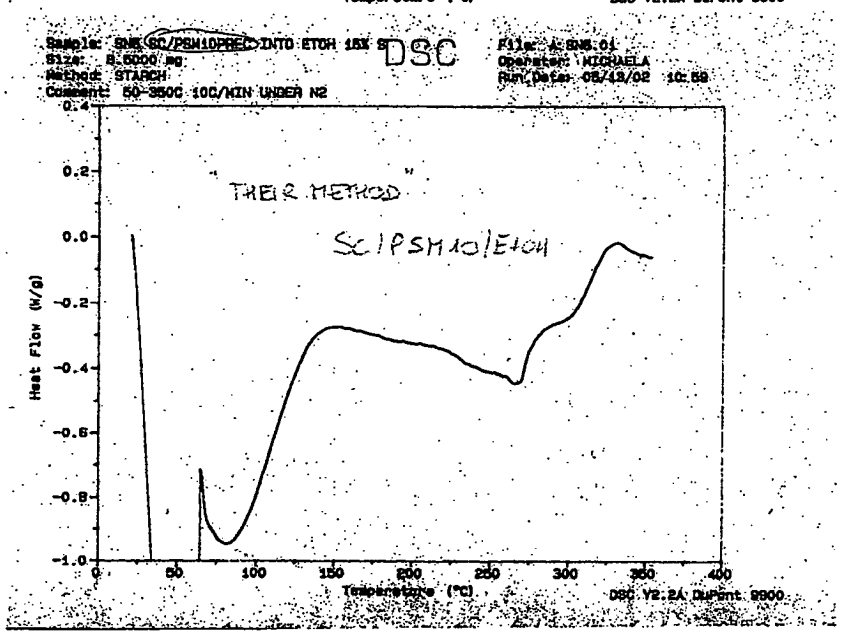
DSC-results

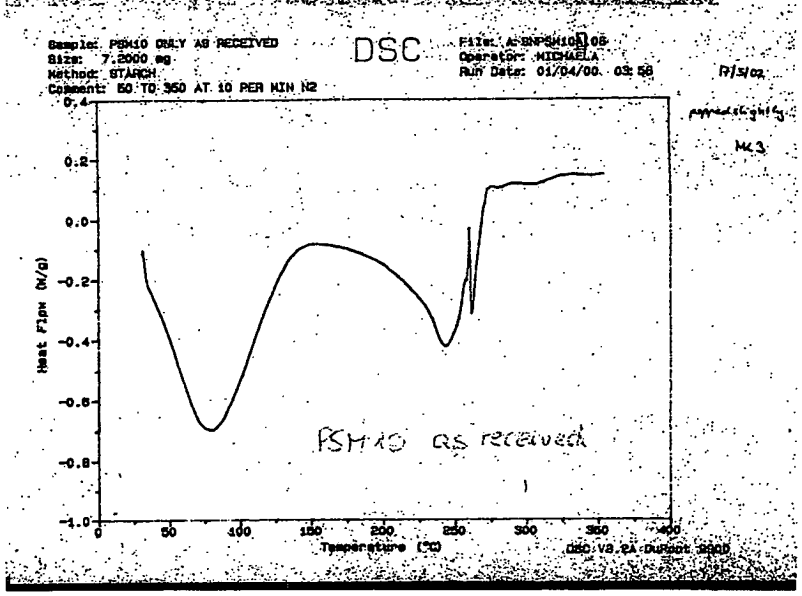
FIG 1B: PSM 10

Fig. 1B









DSC-results

FIG 1C PASELLI STARCH

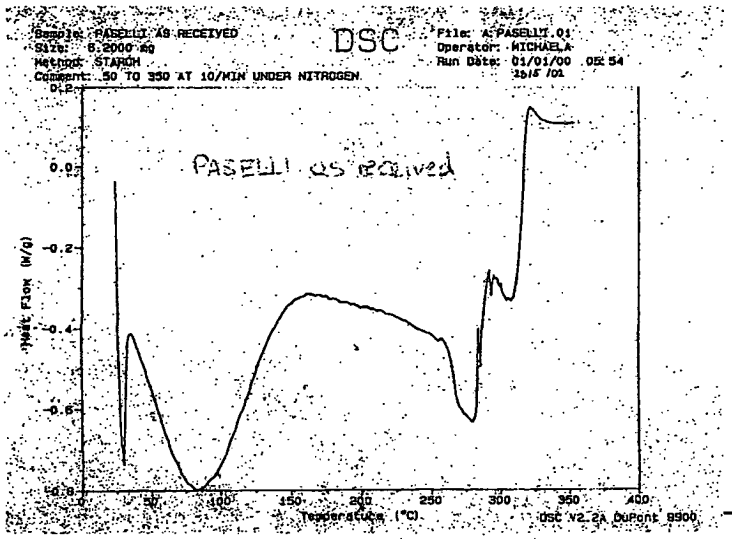
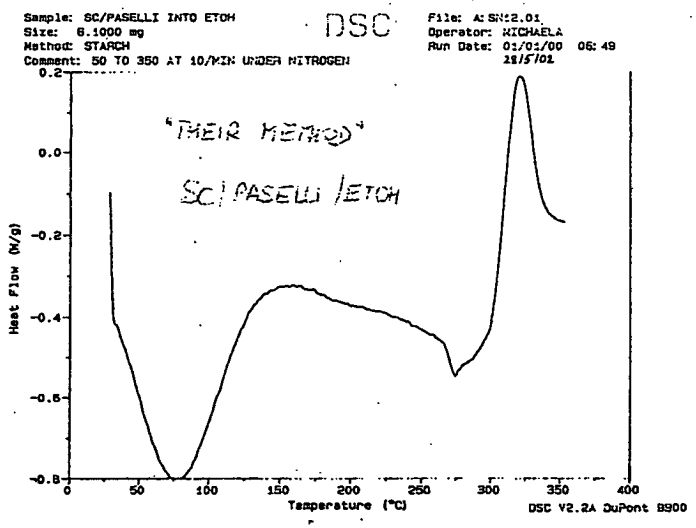
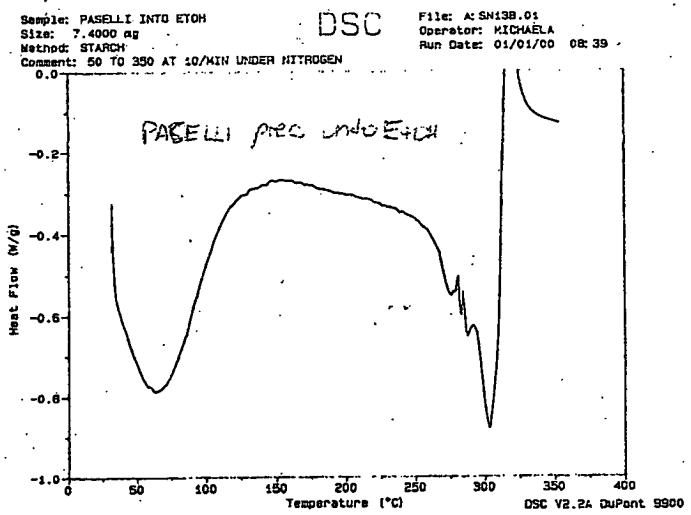


Fig. 1c

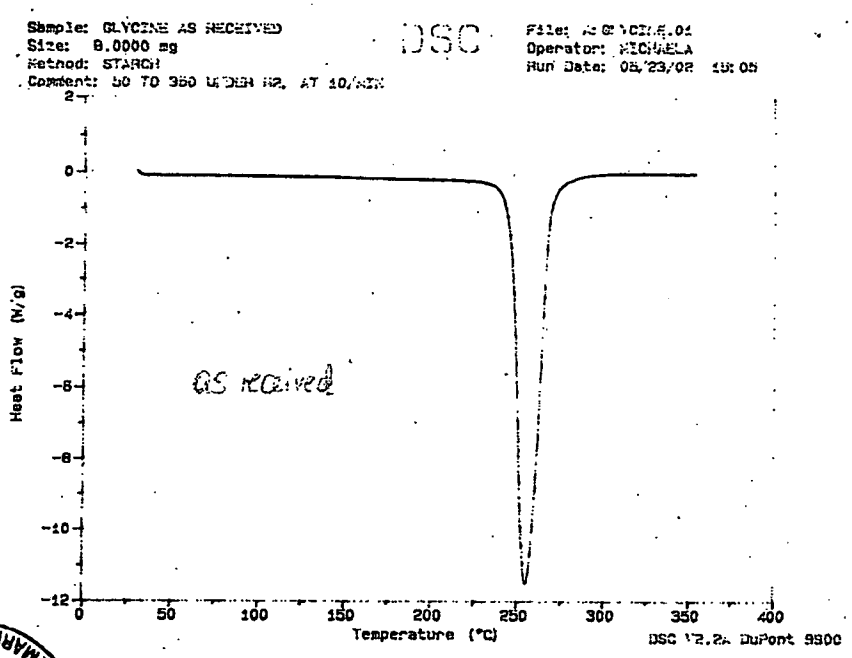




DSC RESULTS

Fig 1D GLYCINE (CONTROL

Fig. 1D



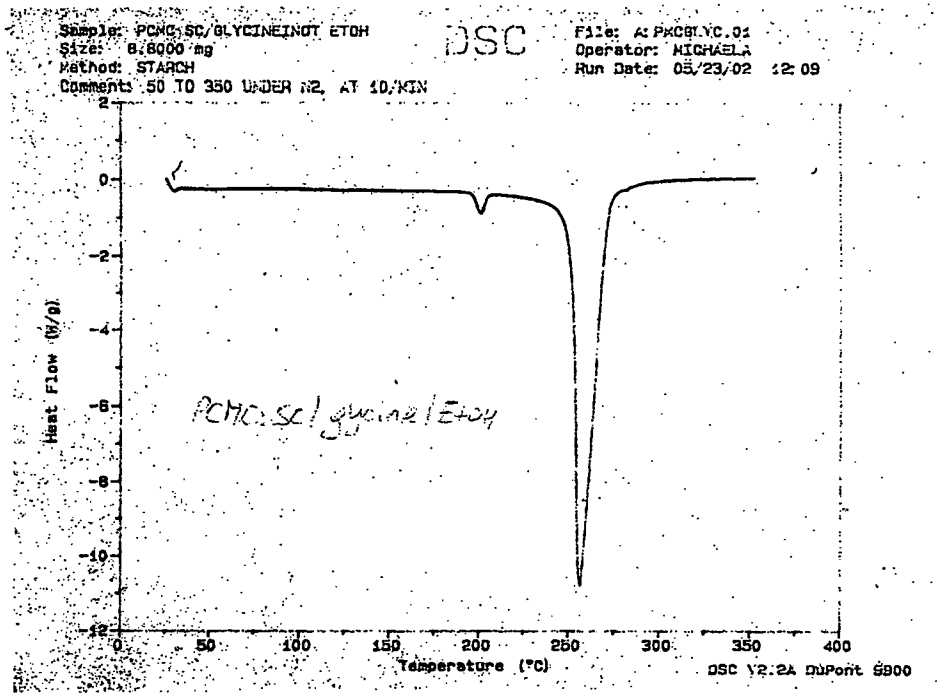
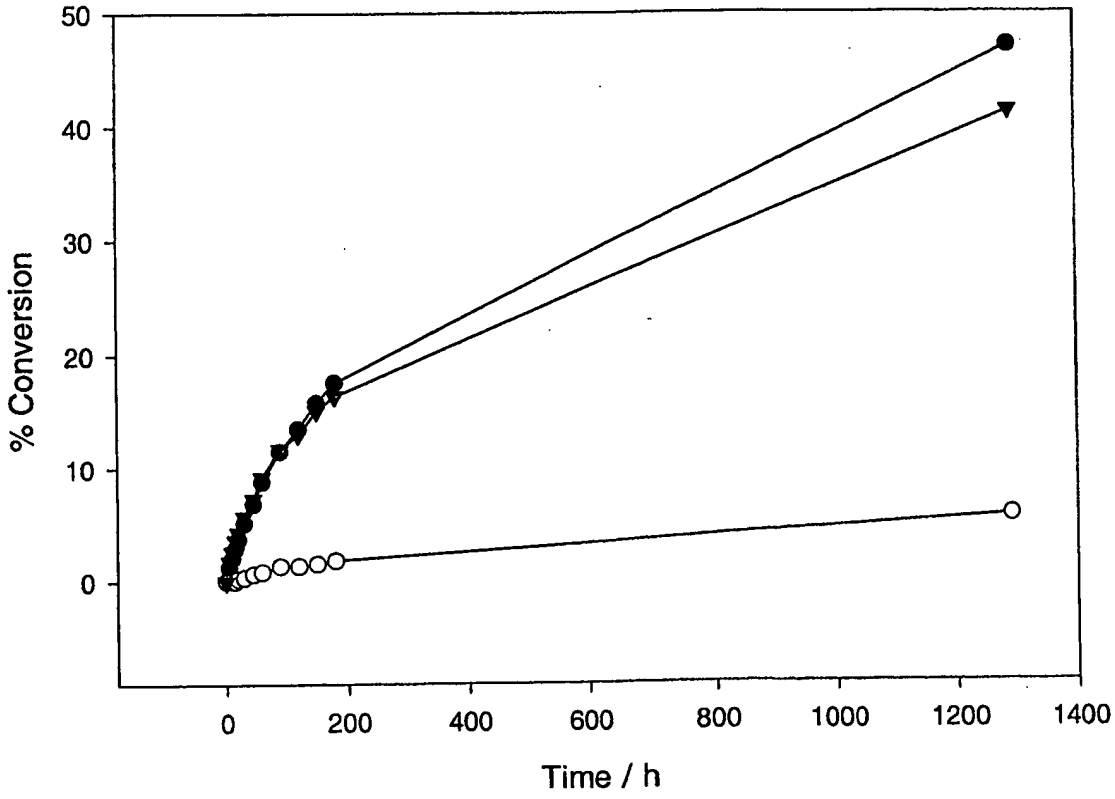


Fig. 2A

STARCH as excipient for SC-PCMCs

Theoretical Loading: 10.8%

Transesterification o N-Ac-Tyr ethylester in AcCN-1%H_2O



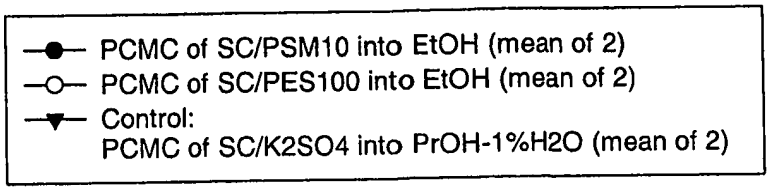


Fig. 2A

Fig. 2B

Starch as excipient for lipases

Pseudomonas cepacia lipase

Kinetic resolution of phenylethanol in dry tert-butyl methyl ether

1.5 mg lipase per reaction (R133)
(2 reaction per condition)

ETOH/bottle as precipitating solvent

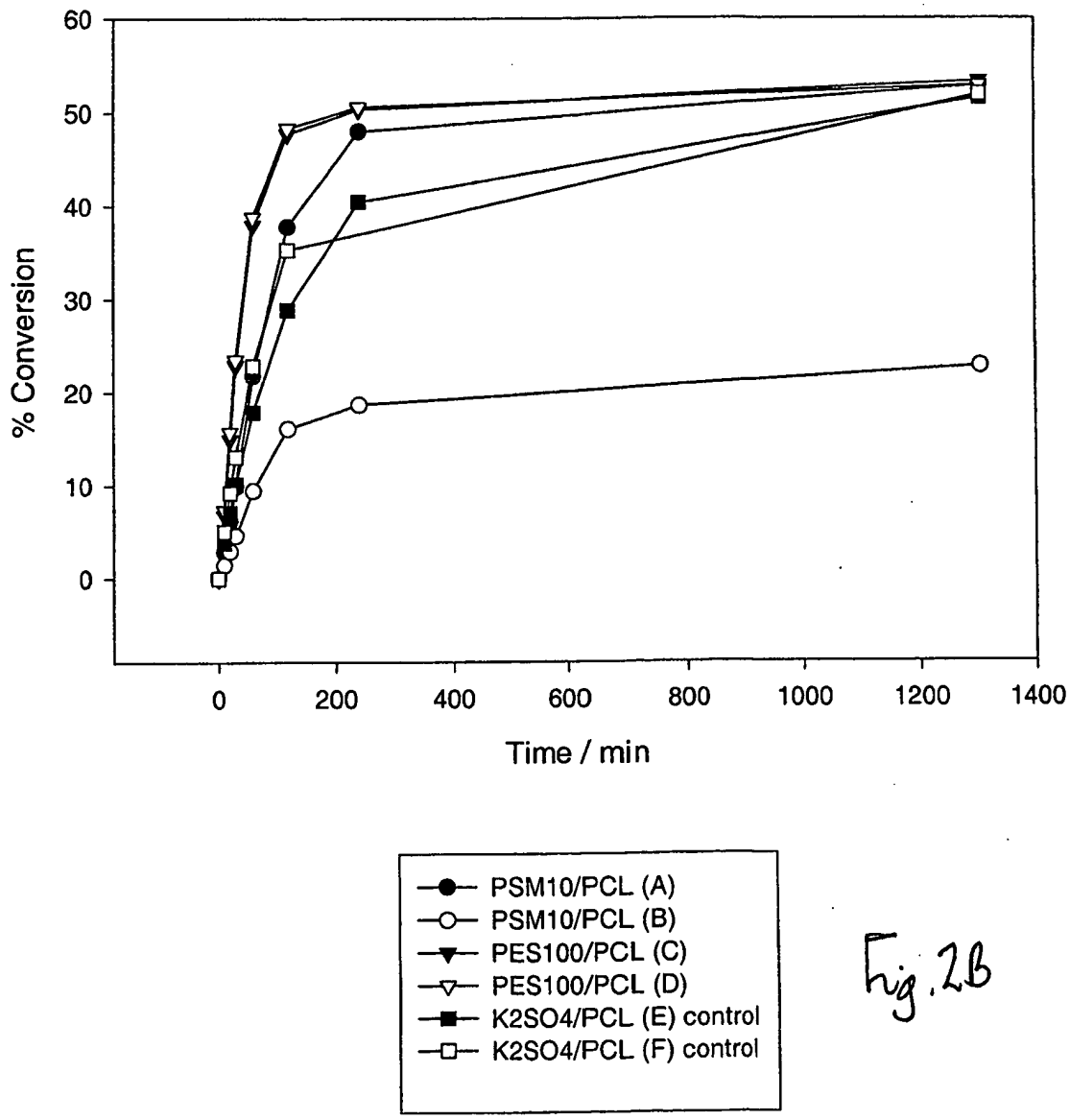


Fig. 2B

Fig. 3A DVS of PES100 starch as received

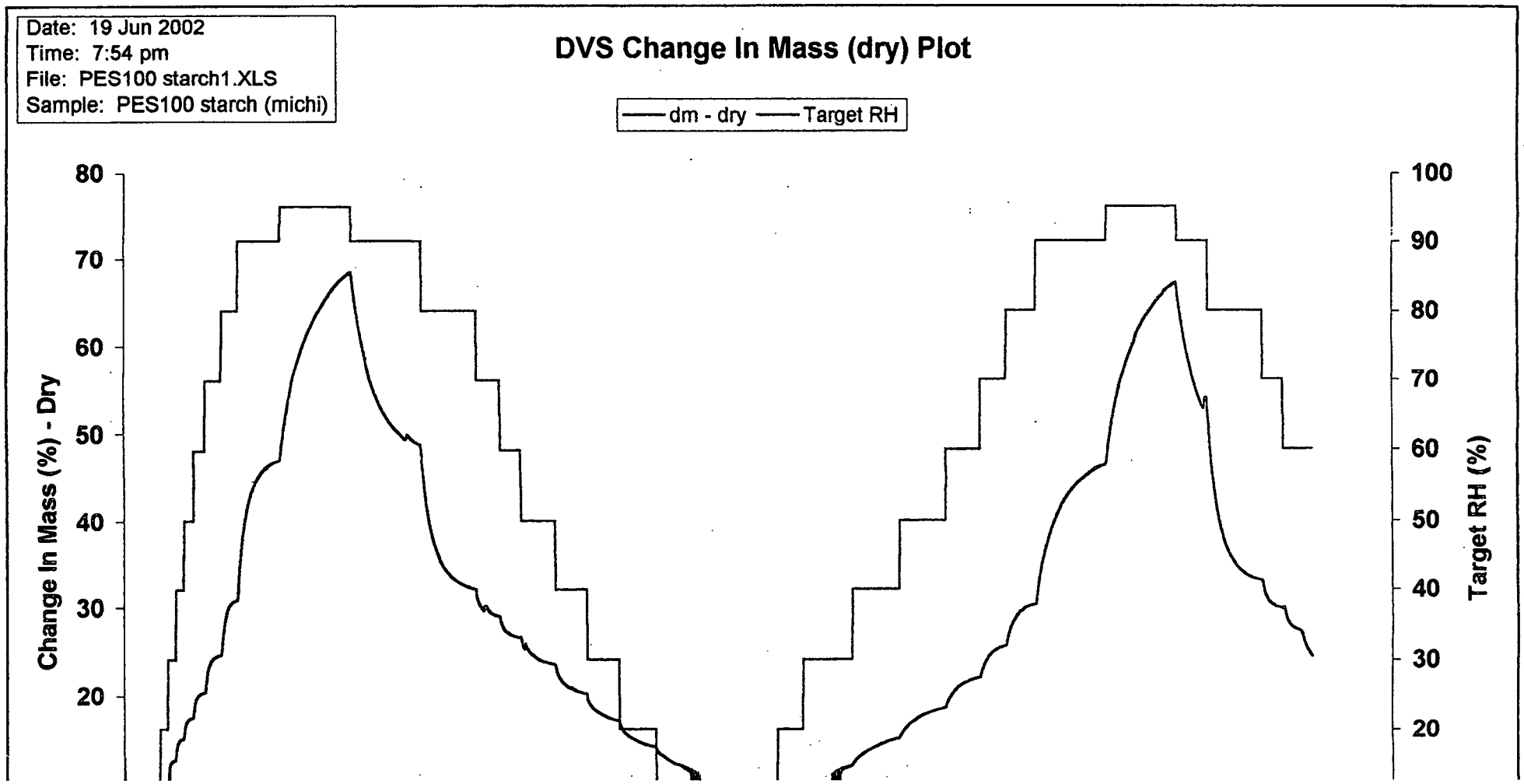


Fig. 3A

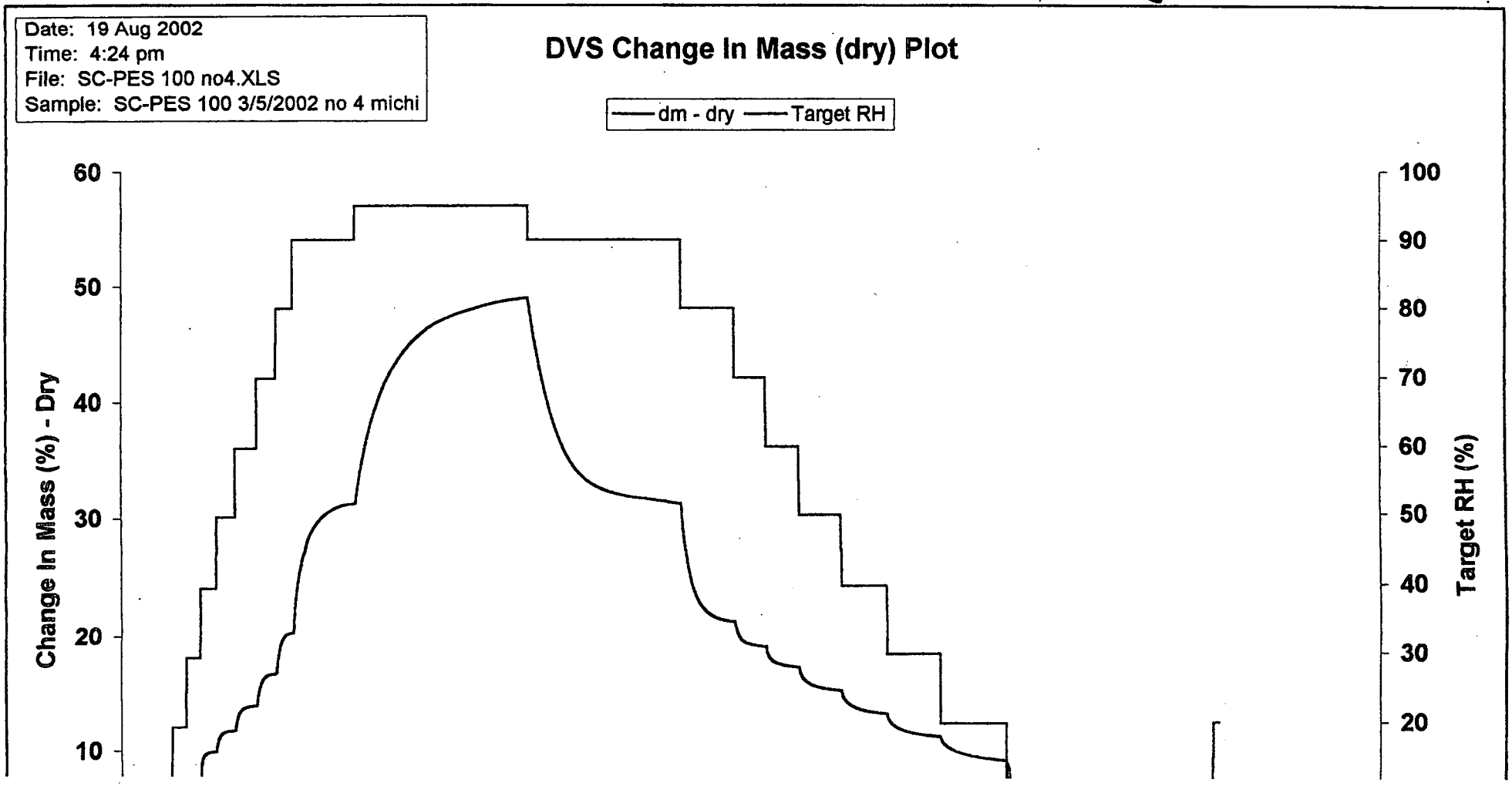
fig. 3A (contd.)

DVS Isotherm Analysis Report

Date: 19 Jun 2002
Time: 7:54 PM
File: C:\Program Files\dvswin 2_16\data\ali\PES100 starch1.XLS
Sample: PES100 starch (michi)
Temp: 26.4 °C

	Target RH (%)	Change In Mass (%) Sorption	Desorption	Hysteresis
Cycle 1	0.0	0.00	6.86	
	10.0	6.29	11.03	4.74
	20.0	9.60	13.88	4.29
	30.0	12.32	16.98	4.66
	40.0	14.83	20.17	5.34
	50.0	17.37	23.47	6.09
	60.0	20.34	26.51	6.17
	70.0	24.54	28.99	4.45
	80.0	30.82	32.04	1.22
	90.0	46.89	48.62	1.72
	95.0	68.49	68.49	
Cycle 2	0.0	6.86		
	10.0	7.45		
	20.0	8.94		
	30.0	11.73		
	40.0	14.93		
	50.0	18.54		
	60.0	22.00	24.36	2.36
	70.0	25.59	29.75	4.17
	80.0	30.26	32.98	2.72
	90.0	46.20	53.84	7.64
	95.0	67.20	67.20	

Fig. 3B DVS of SC/PES100/EtOH - co-precipitate (preparation No. 4 in TABLE 1)



DVS Isotherm Analysis Report

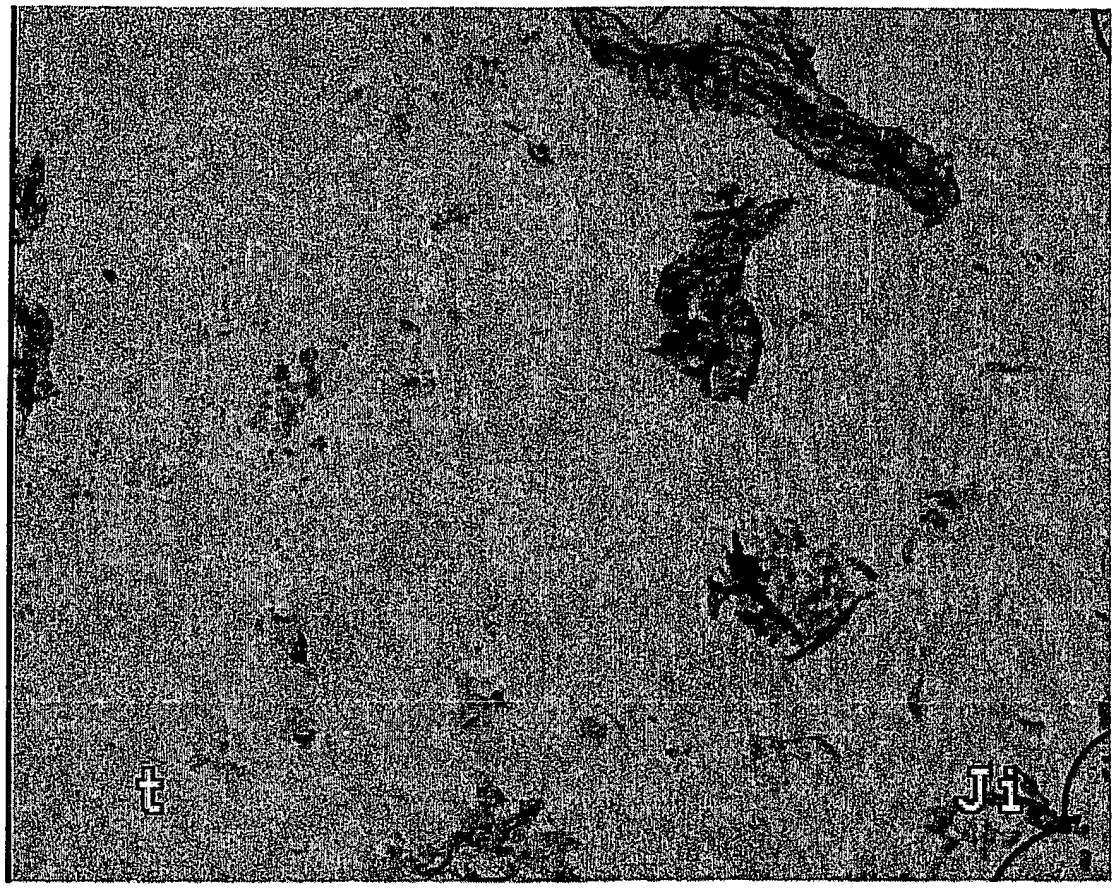
Date: 19 Aug 2002
Time: 4:24 PM
File: C:\Program Files\dvswin 2_16\data\ali\SC-PES 100 no4.XLS
Sample: SC-PES 100 3/5/2002 no 4 michi
Temp: 26.1 °C

	Target RH (%)	Change In Mass (%) Sorption	Desorption	Hysteresis
Cycle 1	0.0	0.00	4.45	
	10.0	4.24	6.67	2.43
	20.0	6.39	8.73	2.34
	30.0	8.15	10.81	2.66
	40.0	9.84	12.85	3.00
	50.0	11.65	14.89	3.24
	60.0	13.83	16.94	3.11
	70.0	16.59	18.73	2.14
	80.0	20.14	20.95	0.81
	90.0	31.11	31.01	-0.10
	95.0	48.92	48.92	
Cycle 2	0.0	4.45		
	10.0	5.02		
	20.0	5.40		

DVS - The Sorption Solution

Fig. 4a PES100 preparations

Fig. 4a



PES 100 only 4x

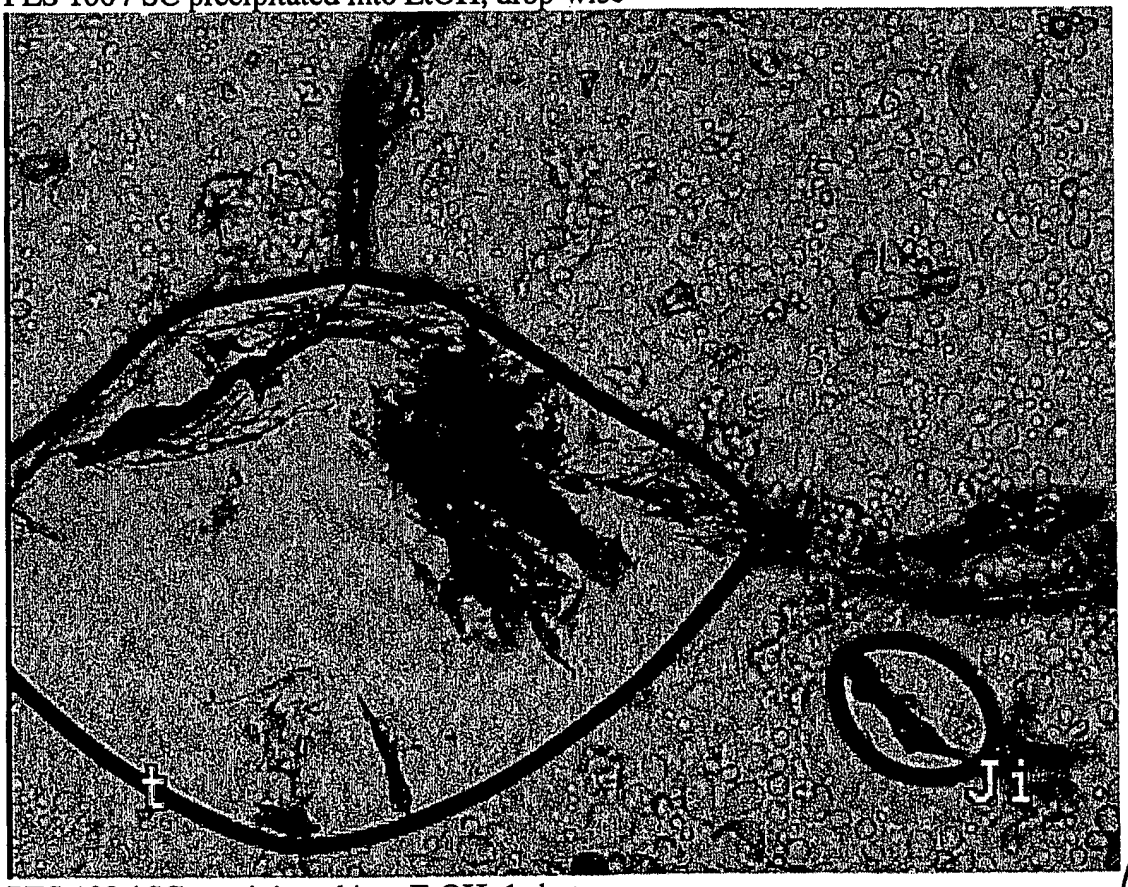
PES 100 PCMC

100µm

Fig. 4b PES100 preparations

Fig. 4b

PES 100 / SC precipitated into EtOH, drop-wise

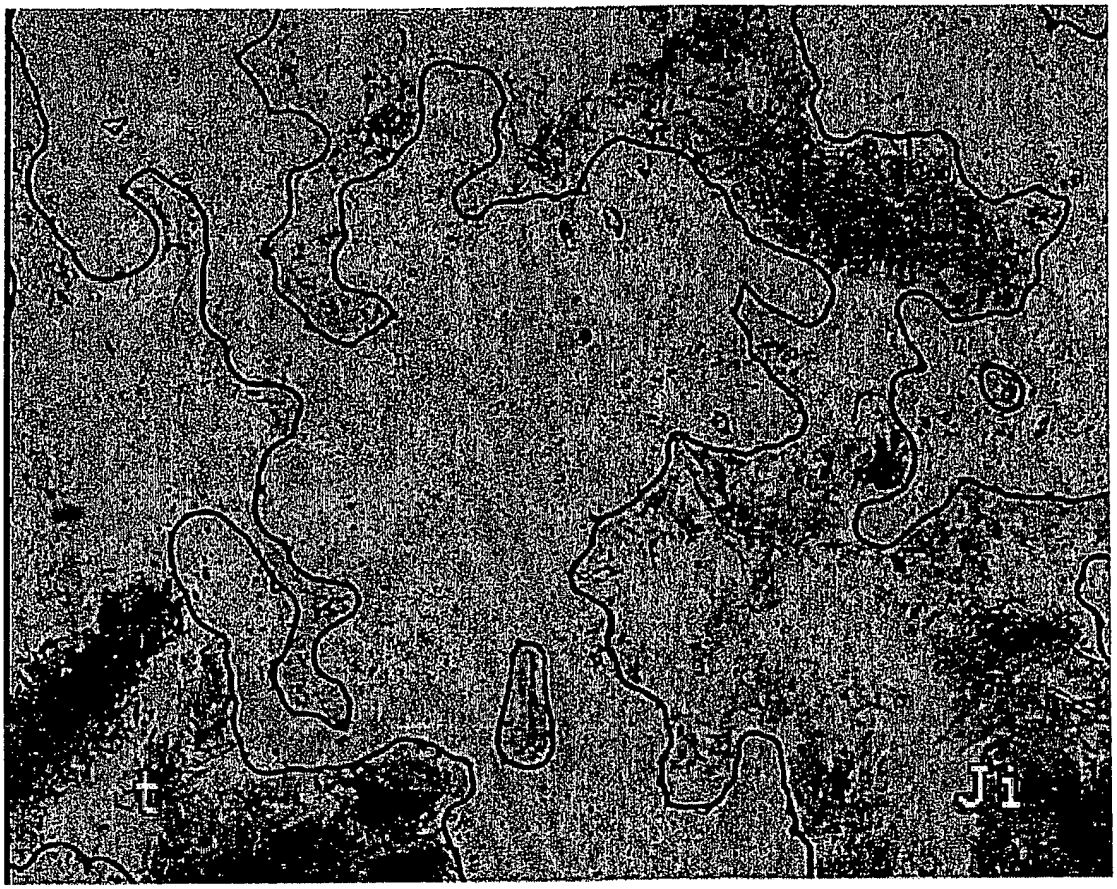


PES 100 / SC precipitated into EtOH, 1 shot

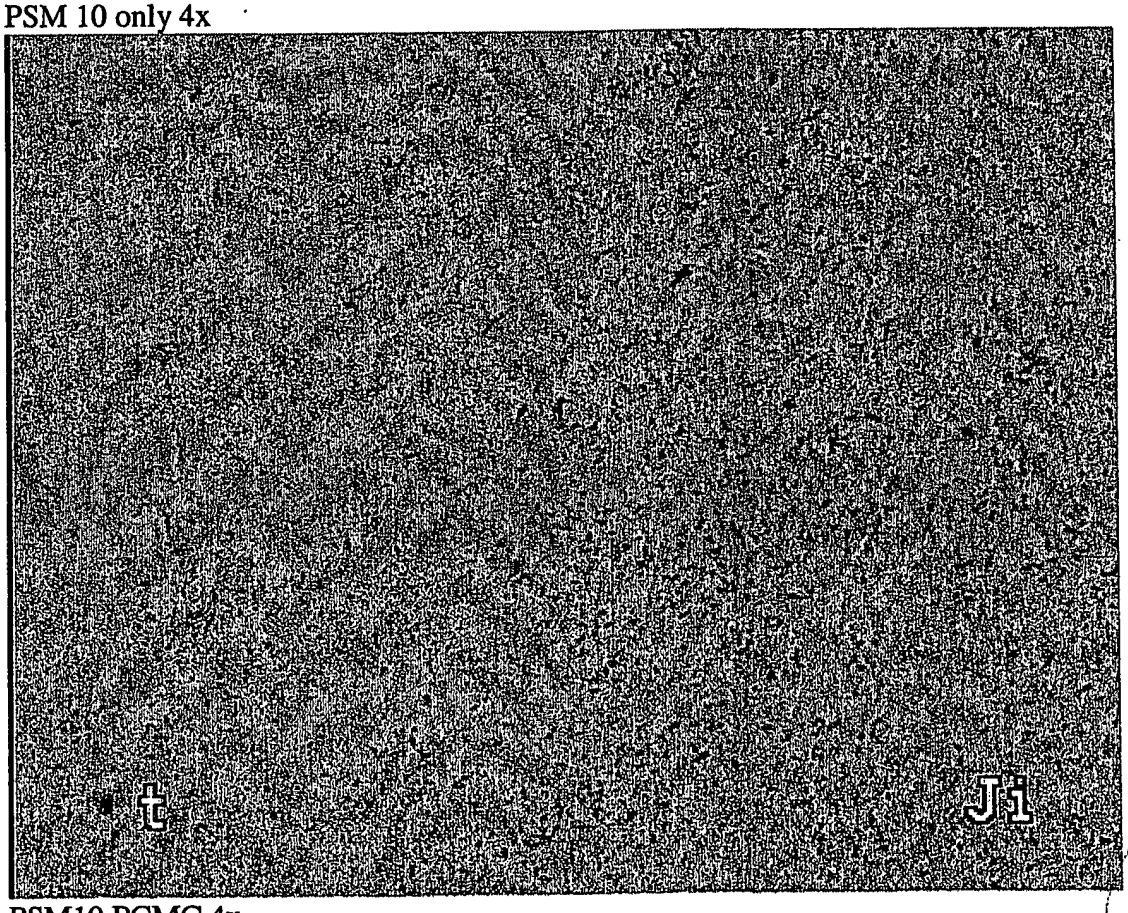
100µm

Fig 5a PSM 10 preparations

Fig. 5a



PSM 10 only 4x

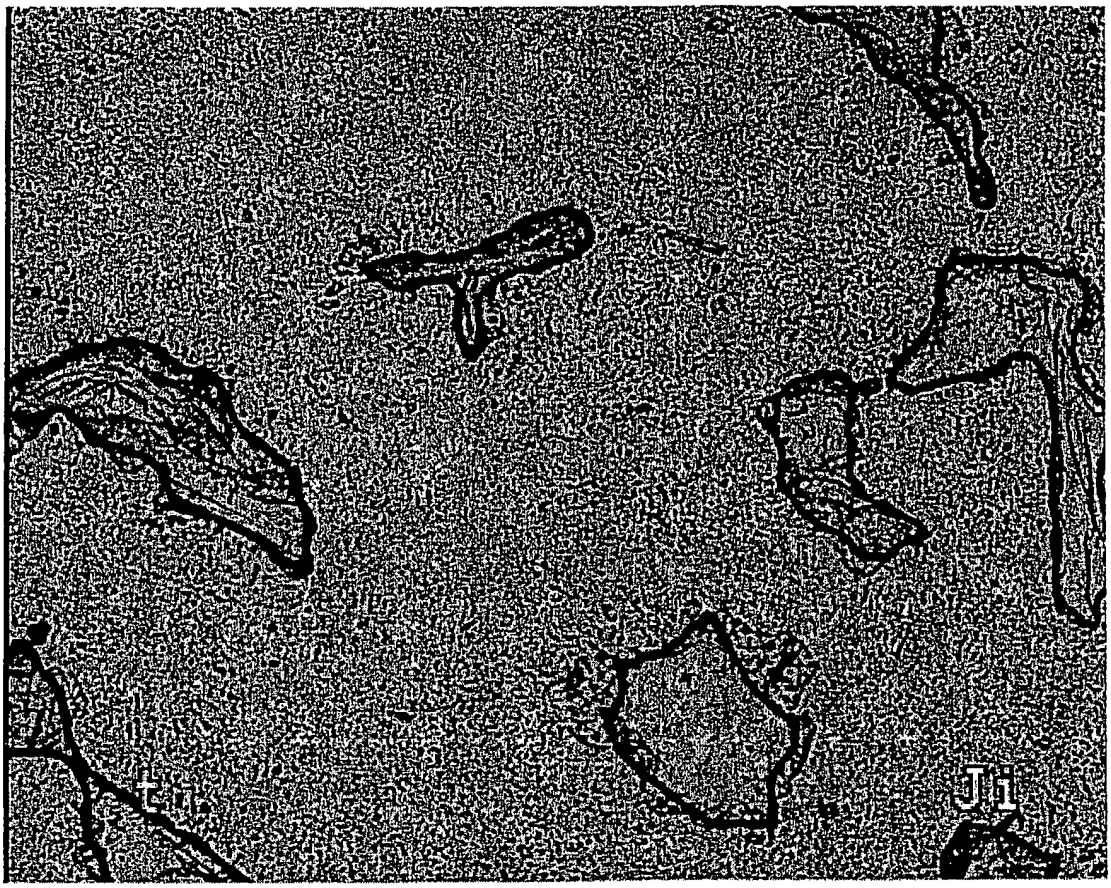


PSM10 PCMC 4x

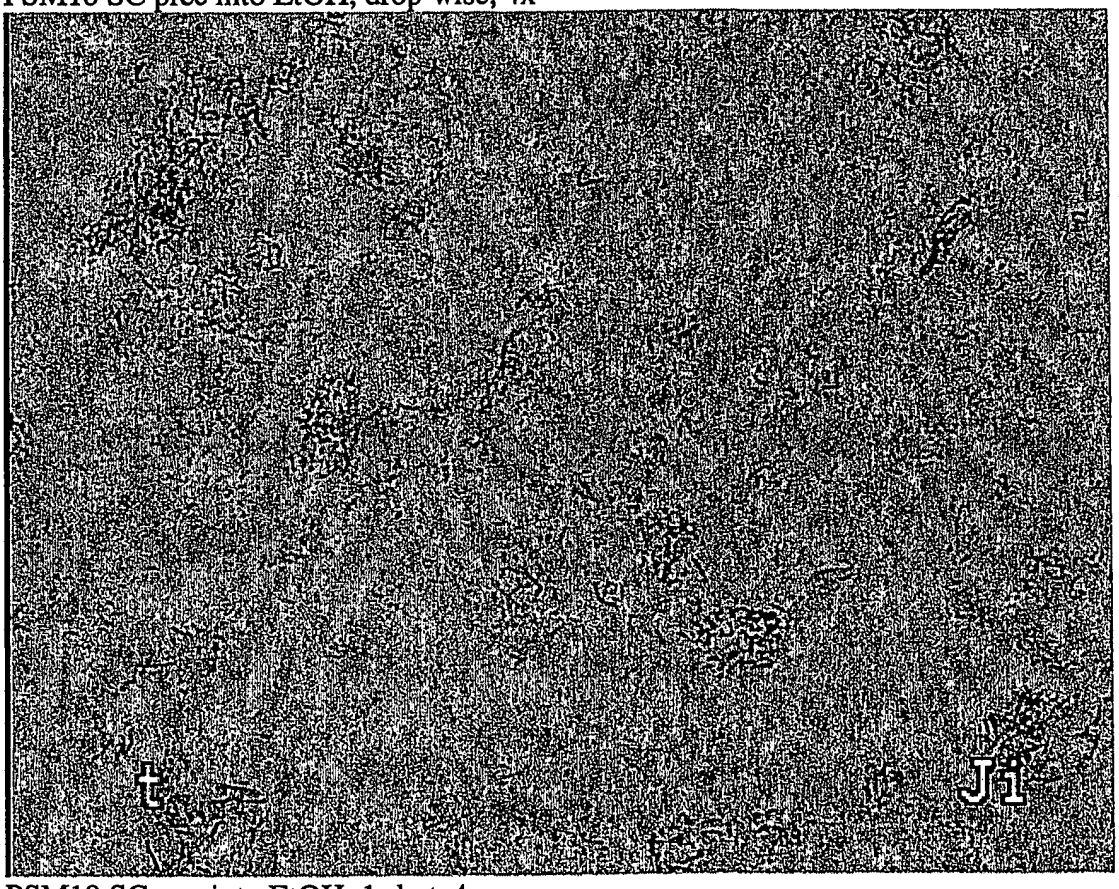
100μm

Fig 5b PSM preparations

fig. 5B



PSM10 SC prec into EtOH, drop-wise, 4x



PSM10 SCprec into EtOH, 1 shot, 4 x

100μm

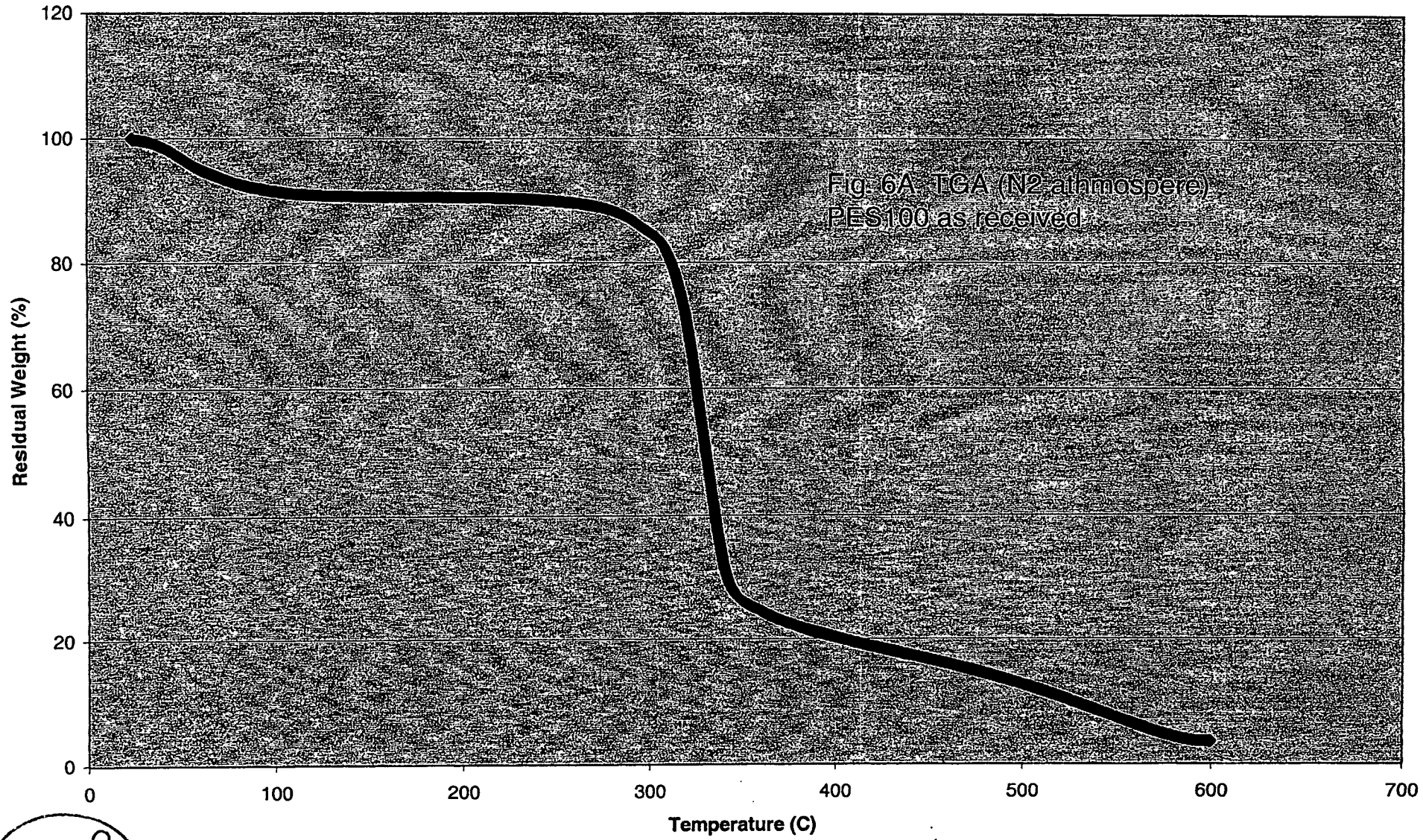


Fig. 6A. TGA (N2 athmospere) PES100 as received

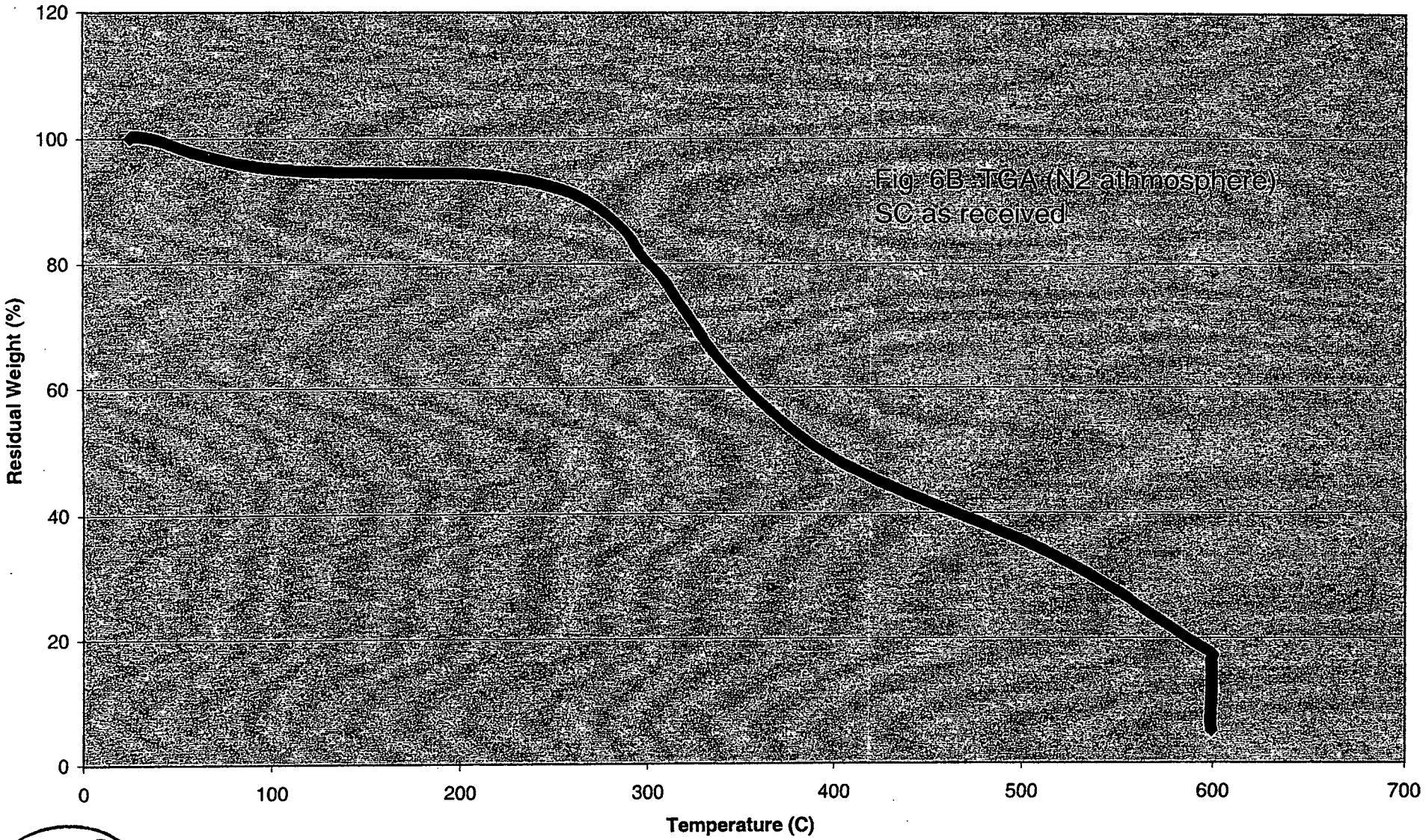


Fig. 6B TGA (N2 athmosphere) SC as received